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The functioning in unanaesthetized sheep of the popliteal lymph node after the surgical removal of its blood supply

J.G. Hall and H.D. Sinnett*
*Divisions of Medicine and *Surgery, Institute of Cancer Research and Royal Marsden Hospital, Downs Road, Sutton, Surrey, UK*

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Summary. Operations were performed to cannulate the efferent duct of the popliteal node of sheep and, at the same time, the blood vascular system was removed surgically from the popliteal fossa so that the node was deprived of it blood supply. Twelve preparations were technically successful in that lymph flowed spontaneously from the unanaesthetised sheep for from 3 to 30 days after the operation. Eight control preparations were established in which the blood supply of the node with the cannulated efferent duct was left intact. In only four of the test preparations was the function of the node decisively impaired so that dendritic macrophages appeared in the lymph, the output of lymphocytes remained very low, and later histological examination showed the nodes to be grossly depleted of lymphocytes. In two of these four preparations the surgical devascularization of the node was aided by arterial embolization. In the remaining eight test preparations the outputs of lymphocytes in the lymph gradually regained normal values, and the nodes then responded normally to antigenic stimuli.

Keywords: lymph node, blood supply, functioning, sheep

It has been noted that, in dogs, the popliteal lymph node was able to survive complete occlusion of its blood supply, provided that a normal flow of lymph through the node was maintained (Holman & Self 1938; Tilak & Howard, 1964). More recently, Osogoe and Courtice (1968) made acute collections of lymph efferent from the popliteal nodes of anaesthetised rabbits, at various times after the nodes' blood supply had been occluded by the injection into the femoral arteries of a latex mass. They found that although the outputs of lymphocytes from the nodes were reduced for a few days after the treatment, the nodes soon became revascularized and able to populate the lymph with normal numbers of lymphocytes.

The notion of a devascularized node, maintained and nourished by nothing more than the lymph which flows through it, has a number of attractions for the experimentalist. For example, such a preparation could provide an absolute zero value in the context of the measurement of blood flow; or, because of the exclusion of recirculating lymphocytes, it could provide an immunolo-

Correspondence: Professor J.G. Hall, Block X, Institute of Cancer Research, Downs Road, Sutton, Surrey, SM2 5PX, UK.

gically privileged site for the growth of allogeneic tumours or hybridoma cells.

In order to pursue such possibilities we have sought, by surgical means, to deprive the popliteal nodes of sheep of their blood supply. The detailed anatomy of this blood supply has been documented already (Heath & Brandon 1983) and the methods for cannulating the efferent duct and monitoring the cellular efferent and immunological functions of the node are well established (Hall & Morris 1962; 1963; Hall 1971).

Materials and methods

General experimental design. At operation the efferent duct of the popliteal node was identified and cannulated so that the lymph coming from the node could be collected quantitatively. When, and if, the cannulation had been carried out successfully the blood vascular system in the popliteal fossa was exposed by careful dissection and all the major vessels were ligated, divided and removed; smaller vessels were destroyed by diathermy. The aim was to engineer a situation in which the popliteal node was attached to the animal by nothing more than its afferent and efferent lymphatic ducts. After the devascularization of the fossa had been completed the wound was closed and the sheep was returned to its pen. The heparinized bottle in which the lymph was collected was changed two or three times each 24 h and the volume, total and differential white cell count were measured and recorded. Preparations in which the lymph did not flow freely for at least 3 days were excluded from the series. In those preparations which flowed for substantial periods of time it was possible to assess the immunological function of the nodes by injecting an antigen s.c. into the lower part of the leg and noting the later efflux of immunoblasts and specific antibody into the lymph (Hall 1971).

Animals and surgical procedures. Crossbred ewes and wethers weighing approximately 30 kg were obtained from local livestock auctions and kept in individual 1 × 1.5 m pens in a purpose-built sheep house. They were fed a standard ration of hay and rolled oats with water and mineral lick *ad libitum*. The sheep were starved for 24 h before being operated on, under aseptic conditions, in a properly equipped theatre. Anaesthesia was induced with i.v. pentobarbitone sodium and maintained by an oxygen–halothane mixture administered via a cuffed endotracheal tube which was connected to the closed circuit of a Boyle anaesthetic machine.

The details of the surgical approach to the popliteal fossa and the cannulation of the efferent duct of the popliteal node have been described (Hall & Morris 1962). The additional procedure in the present study was the removal of the vascular supply to the node. This could not be accomplished by the mere ligation of the hilar vessels (even if these could be found without damaging the afferent lymphatics) because small accessory vessels enter the node at many points over its entire surface (Heath & Brandon 1983). Instead, we adopted the practice of removing as much vascular tissue from the fossa as possible and this is illustrated diagramatically in Fig. 1. The circumflex femoral artery enters the superior angle of the fossa and supplies its contents. Usually, it branches into two major vessels; one of which supplies biceps femoris, the other going to semi membranosus. Commonly, a branch from the artery supplying the biceps courses to the general region of the node and gives rise to the hilar artery. However, the exact disposition of all these structures varied a great deal, even between the two legs of the same sheep, and since they were all embedded in fat a great deal of painstaking dissection was necessary to remove them without damaging the efferent or afferent lymphatic ducts. The majority of the latter enter the inferior angle of the fossa with the lateral saphenous vein and it was fairly easy to preserve them 'en bloc'. In order to make sure that the node was genuinely free of connections with deep surface of the fossa it was often convenient to remove completely the lateral saphenous

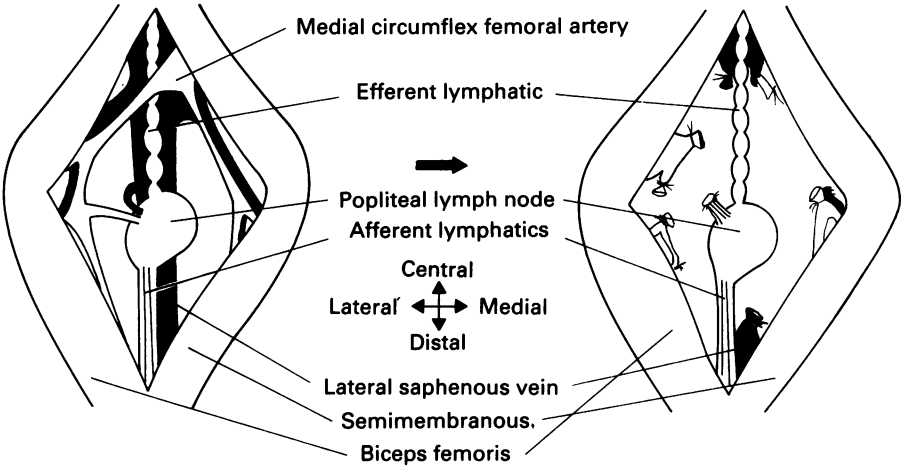

Fig. 1. Diagram to show the position of principal vascular structures within the popliteal fossa before and after the surgical devascularization of the popliteal node.

vein, the largest single vascular structure in the fossa.

In some experiments the major arterial supply to the node was sufficiently obvious and accessible for direct cannulation and on two occasions this was done so that the blood supply to the node could be occluded by embolization. To this end, 2 to 3 ml of a 25% suspension in saline of homologous lymphocytes that had been fixed with glutaraldehyde was infused into the arterial cannula.

Cell counts and histology. Total white cell counts were performed optically using a Neubauer chamber filled with lymph that had been diluted appropriately with 2% acetic acid. Differential white cell counts were made by direct microscopy of a film of untreated lymph under a cover slip using × 100 objective and phase contrast optics. Occasionally, when the white cell count was very low it was necessary to concentrate the cells by a prior centrifugation and resuspension.

Tissues for histology were fixed for 24 h in 0.15 M cacodylate buffer, pH 7.4, containing 2% of formalin and 0.2% of glutaraldehyde. The fixed tissues were processed, embedded in wax, sectioned and stained by conventional methods.

Expression of results and assessment of lymph node function. The lymphocytes in the efferent lymph of a peripheral node like the popliteal are derived almost entirely from the blood passing through the node (Hall & Morris 1964; 1965; Hall 1967). This ability of the node to transmit lymphocytes from blood to lymph is measured in terms of the lymphocyte output in the efferent lymph, a figure obtained by multiplying the flow rate of the lymph (ml/h) by its lymphocyte count. Under basal conditions the lymphocyte output of the popliteal node of unanaesthetized sheep is about 30×10^6/h/g node wt (Hall 1967), although it is depressed by steroid-mediated stress for 20 h or so after anaesthesia and operation (Hall & Morris 1962). This basal performance is relatively constant in any one preparation but varies widely between individuals. In order to make results from different sheep directly comparable the basal lymphocyte output of each preparation was converted to correspond to an arbitrary figure of 100.

When lymph node is severely damaged by

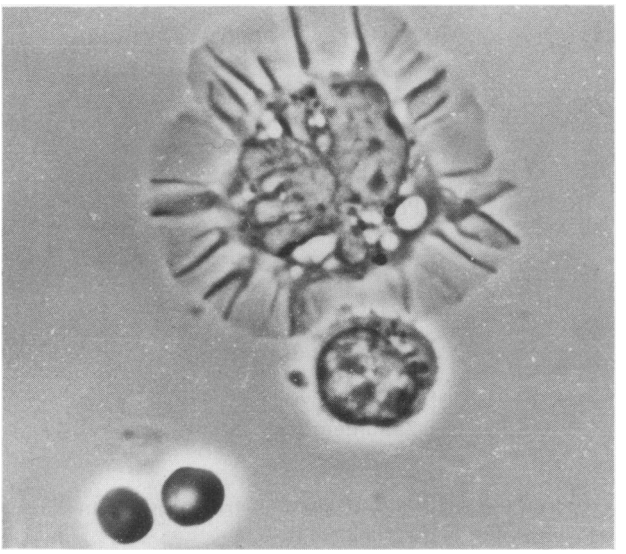

Fig. 2. Phase contrast photomicrograph (approx. × 1700) of a dendritic macrophage in sheep lymph to show the ebullient hyaloplastic membrane and endocytic vesicles characteristic of the living cell. A small lymphocyte and two red cells are shown also. The occurrence of dendritic cells in efferent lymph is a sure sign of damage to the node.

being frozen by a liquid nitrogen probe (Hall & Scollay, unpublished observations 1975) or by toxic chemicals (Hall 1984) dendritic macrophages appear in the lymph. These dramatically obvious cells (Fig. 2) are normally present in the peripheral lymph which is conveyed to the node by the afferent lymphatics but, under physiological conditions, they are retained by the node and rarely gain access to the efferent lymph (Morris *et al.* 1968; Smith *et al.* 1970). Thus the appearance of dendritic macrophages in countable numbers in the efferent lymph is a sure sign of a major impairment of the functional integrity of a lymph node.

Antigens and antigenic stimulation. In order to stimulate the popliteal node of sheep and provoke the changes characteristic of an immune response in the efferent lymph, a suspension of antigens was injected s.c. into the lateral aspect of the leg between the fetlock and the hock; materials injected in this way go principally to the popliteal node (Hall & Morris 1963). In the present study a mixture of two antigens was used. It was composed of 2 ml of an agglutinable suspension of killed *Brucella abortus* (Wellcome Research Laboratories, Beckenham, Kent) and 2 ml of a human influenza vaccine ('Influvac', Duphar Laboratories, Southampton). The antibodies that later appeared in the lymph plasma were titrated by direct bacterial agglutination and haemagglutination inhibition respectively. The titrations were carried out in 0.1 ml systems in conventional, round-bottomed microtitre plates. In the test sheep, the titres of specific antibodies that appeared in the lymph plasma were of the same order as those found in normal sheep. Similarly, antibodies appeared also in extracts of washed lymph cells (Hall & Morris 1963), showing that the specific immune response had taken place in the popliteal node from which the cells had come.

Results

Efferent lymph from the popliteal node was collected from 20 preparations; eight were normal, control preparations in which the

vascular system of the popliteal fossa had been left substantially intact, and 12 comprised the actual experimental group in which the surgical devascularization of the node had been attempted. In both groups the rate of flow in the lymph was rather low in the first few post-operative hours while the recumbent sheep recovered from the effects of anaesthesia (Hall & Morris 1962). At all later times the lymph flowed at between 2 and 8 ml per hour, the flow rate being relatively constant for any one preparation. Thus the observed changes in the output of lymphocytes reflected primarily changes in the count of lymphocytes in the lymph, rather than variations in the flow rate. Generally, there was no sign that the locomotor functions of the legs with devascularized fossae were impaired, and the post operative condition of the experimental sheep did not differ from that of the controls.

The salient results from the experimental group are summarized in Table 1. It can be seen that in eight out of 12 experiments the outputs of lymphocytes ultimately reached substantial values, well within the normal range, inspite of the deliberate surgical damage inflicted on the blood supply to the nodes. However, even in these eight cases it was apparent that the ability of the nodes to transmit lymphocytes from blood to lymph had been impaired significantly, if transiently. This is shown in Fig. 3 where the post-operative recovery of the lymphocyte outputs in these eight experimental sheep is compared with that of controls. In the control group the lymphocyte outputs had all reached their constant, basal level within about 22 h, whereas in the eight experimental preparations it took, on average, more than 5 days for this to happen. In one of these eight cases (No. 7) the function of the node was so impaired initially that the lymph contained significant numbers of macrophages; even so it recovered, so that within a fortnight the large scale recirculation of lymphocytes had been restored. Also, five of the eight preparations flowed long enough to be challenged with antigens; all showed normal cellular and humoral responses.

Table 1. Results from 12 preparations in unanaesthetized sheep in which the flow and cellular composition of lymph efferent from the popliteal node were observed after the attempted surgical removal of the nodes' blood supply.

Preparation number	Days of lymph flow	Output of lymphocytes in millions per hour				Maximum percentage of macrophages	Later response to antigenic challenge
		24 h post op.	48 h post op.	72 h post op.	Maximum attained		
1	30	11	20	25	173	0	N
2	14	5	5	16	100	0	N
3	27	1	24	49	150	0	N
4	7	1	5	12	20	0	nt
5	7	3	3	2	5	10	nt
6	3	5	40	66	70	0	nt
7	15	5	5	7	90	8	nt
8	17	3	3	9	50	0	N
9	30	4	5	12	220	0	N
10*	25	1	1	7	12	10	nt
11*	3	2	1	1	2	2	nt
12	6	1	1	1	3	5	nt

* Blood supply occluded by aterial embolization.
N Normal.
nt Not tested.

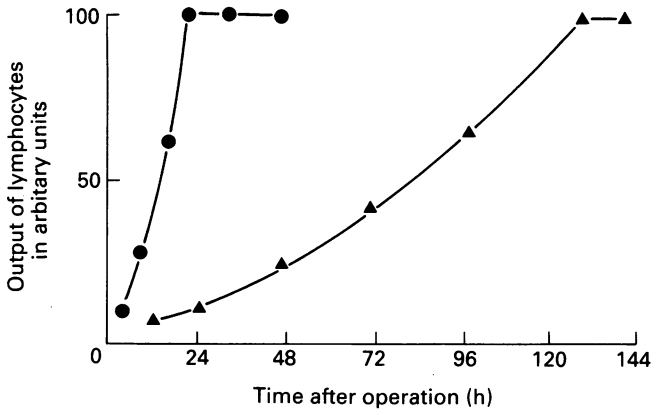

Fig. 3. Graphic comparison of the post-operative recovery of lymphocyte outputs in the lymph from eight test (devascularized) and eight control (normal) popliteal lymph nodes of sheep. Average values are shown and the ultimate basal outputs were converted to correspond to an arbitary figure of 100. Each graph was constructed from over 50 individual observations of lymphocyte output. ●, control; ▲, test.

In the remaining four preparations of the experimental group the function of nodes was grossly impaired for the duration of the experiment. The first of these (no. 5) was terminated deliberately after 7 days so that the node could be examined histologically. A photomicrograph of a section cut from the node is shown in Fig. 4. In comparison with the normal, contralateral node (the efferent duct of which had also been cannulated) the node was grossly depleted of lymphocytes. It was still a substantial structure but was

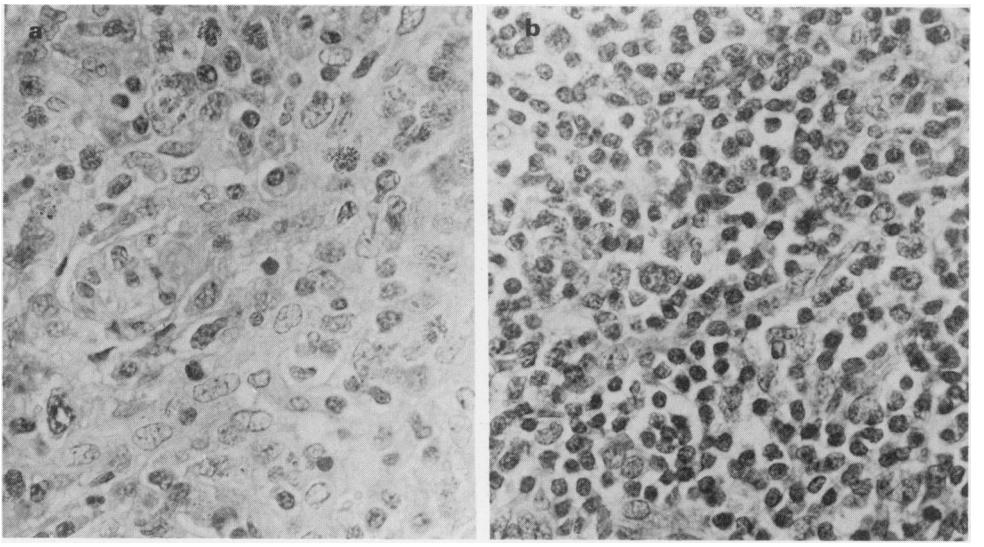

Fig. 4. Photomicrographs (approx. × 420) of sections, stained with haematoxylin and eosin, cut from the popliteal nodes of sheep. *a*, Shows the paucity of lymphocytes in the deep cortex of a node that had been devascularized surgically 7 days previously. *b*, Shows the appearance of the corresponding area of the contralateral, normal (but cannulated) node; it is replete with small lymphocytes. The two photomicrographs were adjacent exposures on the same film (Ilford FP/4).

composed almost entirely of reticulo-endothelial cells with very few lymphocytes and no clearly demarcated cortex, medulla, or follicular structures. The nodes in preparations 10 and 11 were subjected to arterial embolisation before the surgical removal of the blood vessels. Both later yielded typical hypocellular 'peripheral' lymph containing relatively abundant macrophages. Unfortunately, both preparations showed evidence of local peripheral neurophathy as well. In number 10 this resulted in no more than a transient 'foot drop' which resolved spontaneously within 48 h but in number 11 there was a gross flexion deformity of the phalanges so that a pressure sore developed over the 'knuckle' of the fetlock, and the sheep had to be destroyed for humanitarian reasons. Evidently some of the material intended for embolization within the node had gained access to the microvasculature of the nerve trunks which are located on the floor of the popliteal fossa.

In the final sheep in the experimental series (No. 12) we again succeeded in significantly reducing lymph node function by purely surgical means and, again, the node was found to be gravely depleted of lymphocytes on histological examination.

Discussion

The above experiments show clearly that, in sheep, all the major blood vessels of the popliteal fossa can be removed without prejudicing the vitality of the surrounding muscles and skin, which must, presumably, be provided with an adequate collateral circulation. However, inspite of the wholesale vascular clearance of the popliteal fossa, the node, which lies at its centre in a pad of fat, usually became revascularized and regained a normal function. This outcome is in accord with the experiences of Osogoe and Courtice (1968) and suggests that the preparation of a permanently devascularized node will require some special techniques. Also, it is fair to say that we were often surprised that nodes which, in the opinion of two experienced operators, appeared to have been deprived of their blood supply, were nonetheless soon functioning in an entirely normal way. The most obvious explanation for this finding is that revascularization proceeded rapidly from the minute *vasa vasorum* that could be discerned in the adventia of the lymphatic vessels, which we were at pains to leave intact. The whole of the experimental plan was based on maintaining a normal flow of lymph, and this in turn required that the ability of the lymphatic vessels to generate the powerful, intrinsic, rhythmic muscular contractions which force the lymph in a central direction (Hall *et al.* 1965) should be preserved, and for this reason we interfered with the adventitia of the lymphatic vessels as little as possible. Nonetheless, in four experiments there was no sign of any recovery of the lymphocyte output, and macrophages were present in the lymph at all times. It is possible that these nodes might ultimately have recovered their normal functions had spontaneous lymph flow continued for longer than it did but this cannot be known.

The two nodes from these preparations that were examined histologically showed no signs of vascular regeneration and their microscopical appearances were reminiscent of those seen after lethal wholebody irradiation or in severe combined immunodeficiency. Unfortunately, even under normal conditions the lymph nodes of sheep rarely exhibit post capillary venules with high endothelium, that are the site of lymphocyte recirculation in rodents, and so it was not possible to make any particular observation on this functionally important part of the microvasculature.

It is probably significant that neither of the nodes subjected to arterial embolization showed any sign of recovery, and this may point the way to a more successful technique, provided that the side effects can be eliminated.

From a general surgical point of view it seems reasonable to conclude that, given that most of the lymphatic vessels remain

intact, most lymph nodes will be able to recover easily from severe damage to their blood supply.

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